Organic solvent tolerance was tested in type strains of type species of the sixteen genera of Halobacteriaceae, the halophilic archaea. Most of the strains were observed to grow in the presence of hexylether (log $P_{ow}$ = 5.1), but none grew in the presence of n-octane (log $P_{ow}$ = 4.9) except Halogeometricum borinquense JCM 10706T and Halorubrum saccharovorum JCM 8865T. On the other hand, two strains, Haloarcula spp. OHF-1 and 2 isolated from a French solar salt were found to show stronger tolerance even to isooctane (log $P_{ow}$ = 4.8). Growth of some strains was retarded by the presence of n-decane but reached to the same cell densities at late stationary phase. Final cell densities of some strains were greatly repressed by the presence of the solvent.

Key words: halophilic archaea; strains OHF-1 and OHF-2, and log $P_{ow}$ value

The tolerance of microorganisms to organic solvents has been studied extensively since the discovery of the toluene-tolerant Pseudomonas putida strain IH-2000. In fact, the solvent tolerance has been detected in many microbes, and mechanism of tolerance has been investigated.

Extremophiles are microorganisms adapted to conditions of extreme temperature, pH, salinity, etc. Some organic solvents originating from crude petroleum also create natural extreme environments. Recently, a few halophilic strains were isolated from a plot of the Kalamkass oil field filled with saturated brine and oil, and from an estuary polluted with petroleum also create natural extreme environments. Some organic solvents originating from crude oil. No report, however, has been published concerning organic solvent tolerance of representative strains of halophilic archaea. In this report we investigated organic solvent tolerance of type strains of extreme halophiles of the family Halobacteriaceae.

The following type strains of each type species of the sixteen genera were cultivated as follows. Haloarcula vallismortis JCM 8877T, Halobacterium salinarum JCM 8978T, Halococcus morrhuae JCM 8876T, Halofexerax volcanii JCM 8879T, Halogeometricum borinquense JCM 10706T, Halorubrum saccharovorum JCM 8865T, Natronialba asiatica JCM 9576T, Natrinema pellirubrum JCM 10476T, and Halomicrobium mukohataei JCM 9738T were cultivated in JCM medium 168 consisting of Casamino acids, yeast extract, 20% NaCl etc. (http://www.jcm.riken.go.jp). Halobaculum gomorrense JCM 9908T was grown in JCM medium 225, Halorhabdus utahensis JCM 11049T in JCM medium 294, and Haloterrigena turkenica JCM 9101T in JCM medium 169. Alkaliphilic strains Natronobacterium gregoryi JCM 8860T, Natronococcus occultus JCM 8859T, Natronomonas pharaonis JCM 8858T, and Natronorubrum bangense JCM 10635T were cultured in JCM medium 167, which contained 0.5% sodium carbonate to raise the pH to around 9.5. All of these strains are devoid of a peptidoglycan layer, and stain Gram negative, except for Gram-variable Halococcus morrhuae and Natronococcus occultus.

The strains were inoculated in to 2 ml of the liquid media in test tubes and 1 ml of an individual organic solvent (n-decane, n-nonane, hexylether, n-octane, isooctane, or cyclooctane) was added. After vigorous shaking (140 rpm) at 30°C, growth was estimated by measuring the OD$_{660nm}$ of 80 µl of cultures. In some experiments, cultures were done in 100-ml Erlenmeyer flasks containing 20 ml of medium, overlaid with 10 ml of solvent, shaken at 110 rpm at 30°C. Samples were taken periodically to measure OD$_{660nm}$.

Tolerance of bacteria, including archaea, to solvent has been estimated by the solvent parameter log $P_{ow}$ (common logarithm of the partition coefficient of a given solvent in a mixture of n-octanol and water), which is an index of biological toxicity. Lower values represent higher toxicity. Table 1 shows the growth of strains in the presence of various organic solvents. All strains of halophilic archaea showed good growth in media containing n-decane, while most type strains showed no growth in the presence of organic solvents which had log $P_{ow}$ value, below 4.9, except H. borinquense JCM 10706T and H. saccharovorum JCM 8865T. Gram-negative bacteria, Escherichia coli and P. putida for example,
have been shown to be tolerant up to log $P_{sw}$ of 3.8 (propylbenzene) and 3.1 (p-xylene), respectively. Thus, the organic solvent tolerance of the halophilic archaea seems to be weaker than that of Gram-negative bacteria.\(^{21}\)

Growth of some strains was measured periodically in the presence of n-decane and two patterns were obtained. In pattern A, the growth rate at exponential phase was considerably lower in the presence of n-decane (65–75\% of that in the absence of n-decane). In pattern B, both growth rate and the final cell density were low in the presence of n-decane (65–75\%). Representative growth curves of \textit{N. bangense} (pattern A) and \textit{N. gregoryi} (pattern B) are shown in Fig. 1. Other strains tested are also divided into patterns A and B (Table 1). Differences in growth patterns in the presence of n-decane may suggest there is a difference between these two patterns in the expression of genes responsible for the solvent tolerance. Tolerance may be induced in the presence of organic solvents in pattern A, while not in pattern B.

To investigate if there exist any halophilic archaea with stronger solvent tolerance, we isolated some extreme halophiles from a commercially available French solar salt (Sel Marin DE NOIRMOUTIER). The salt sample was dissolved in 50 ml of a sterile medium (consisting of Casamino acids, yeast extract, 0.2\% KCl, and 2\% magnesium sulfate, pH 7.4) at a concentration of 25\%, and incubated at 37°C for 3 weeks without shaking. The culture was then spread on an agar plate of the same medium containing 25\% NaCl. Ten colonies were picked up at random and were tested for the solvent tolerance as described above. Two isolates, OHF-1 and OHF-2, showed tolerance to iso-octane (log $P_{sw}$ = 4.8), while the other eight isolates showed tolerance only up to hexylether (log $P_{sw}$ = 5.1).

To identify OHF-1 and OHF-2, the 16S rRNA encoding genes were amplified by PCR with the following forward and reverse primers: 5’-ATTCCGGTTGATCTCATG (positions 6–25 in \textit{E. coli} numbering) and 5’-AGGAGGTGATCCAGCCGCAG (positions 1540–1521). The amplified 16S rDNAs were cloned into pCR2.1 T-vector (Invitrogen) and sequenced using the Big Dye Sequencing Kit (Applied Biosystems) by the ABI 377 DNA sequencer (Applied Biosystems). Sequencing primers used were 1: 5’-AGGAGGTGATCCAGCCGCAG (positions 1540–1521), 6: 5’-ATTCCGGTTGATCTCATGCG (positions 6–25 in \textit{E. coli} numbering), 7: 5’-AGGAGGTGATCCAGCCGCAG (positions 1540–1521), 7: 5’-ATTCCGGTTGATCTCATGCG (positions 6–25 in \textit{E. coli} numbering), 8: 5’-ATTGGGCCAGTATCATG (positions 563–585), 9: 5’-ATTGGGCCAGTATGATG (positions 563–585), and 10: 5’-GGAAACAGCTATGACCATG (vector side’s primer) and Rev: 5’-GGAAACAGCTATGACCATG (vector side’s primer). The sequences of strains OHF-1 and OHF-2 had very high similarities with that of \textit{Haloarcula argentinensis}, 99.1\% and 99.3\%, respectively, suggesting that the two strains belonged to \textit{Har. argentinensis}. DDBJ accession numbers of OHF-1 and OHF-2 are AB098536 and AB098337, respectively.

Unexpectedly, the type strain of \textit{Har. argentinensis}, JCM 9737\textsuperscript{T}, showed tolerance only up to 5.1, as do most other type strains of other genera. It was

\begin{table}[h]
\centering
\caption{Organic Solvent Tolerance of Halophilic Archaea}
\begin{tabular}{|c|c|c|c|c|c|c|}
\hline
Strains & Organic solvent & n-decane & n-nonane & hexylether & n-octane & iso-octane & cyclooctane \tabularnewline & log $P_{sw}$ value & & & & & & growth patterns \\
\hline
\textit{Halofexx volcanii} JCM 8879\textsuperscript{T} & ++ & ± & ++ & – & – & – & A \\
\textit{Natriaiba asiatica} JCM 9576\textsuperscript{T} & ++ & ± & ++ & – & – & – \\
\textit{Natrinema pellirubrum} JCM 10476\textsuperscript{T} & ++ & + & ++ & + & – & – \\
\textit{Halogeometricum boriingense} JCM 10706\textsuperscript{T} & ++ & ++ & + & ++ & – & – \\
\textit{Natronomas pharaoisis} JCM 8858\textsuperscript{T} & ++ & – & + & + & – & – \\
\textit{Natronorobrum bangense} JCM 10635\textsuperscript{T} & ++ & + & + & + & – & – \\
\textit{Haloarcula sp. strain OHF-2} & ++ & ++ & ++ & + & – & ± & A \\
\textit{Haloarcula vallismortis} JCM 8877\textsuperscript{T} & ++ & ± & + & – & – & – \\
\textit{Halobacterium salinarum} JCM 8978\textsuperscript{T} & ++ & + & ++ & + & – & – \\
\textit{Haloarcula saccharovorum} JCM 8865\textsuperscript{T} & ++ & ± & ++ & + & – & – \\
\textit{Halococcus morrhuae} JCM 8876\textsuperscript{T} & ++ & + & ++ & + & – & – \\
\textit{Haloterrigena turkmenica} JCM 9101\textsuperscript{T} & ++ & ± & + & – & – & – \\
\textit{Halorhabdus utahensis} JCM 11049\textsuperscript{T} & ++ & ± & – & – & – & – \\
\textit{Halococcus gomorrense} JCM 8859\textsuperscript{T} & ++ & + & – & – & – & – \\
\textit{Natronomallys occulitus} JCM 8877\textsuperscript{T} & ++ & + & + & + & – & – \\
\textit{Haloarcula argentinensis} JCM 9737\textsuperscript{T} & ++ & ± & + & + & – & – \\
\textit{Haloarcula sp. strain OHF-1} & ++ & + & ++ & + & – & ± & B \\
\hline
\end{tabular}
\end{table}

\begin{itemize}
\item ++: good growth, +: ±: growth, -: no growth.
\end{itemize}

This table shows the growth of the sixteen type strains, \textit{Har. argentinensis} JCM 9737\textsuperscript{T} and two isolates OHF-1 and OHF-2, in the presence of various organic solvents. Growth patterns A and B shows the different growth curves show in Fig. 1A and B, respectively.
Organic Solvent Tolerant Halophiles also noted that OHF-1 showed pattern B while OHF-2 showed pattern A, thus there was little correlation between growth patterns and the genera. Strains OHF-1 and 2 maintained their tolerance to organic solvent even after repeated sub-culturing in solvent-free medium, suggesting that chromosomes contain genes for organic solvent tolerance. Further studies of the genomes of type strains may reveal any relationships.

In bacteria, genes relevant to solvent tolerance have been identified, such as ostA of *E. coli* K-12, and ttgR of *P. putida* coding for organic solvent efflux pumps. *Halobacterium* sp. NRC-1 is the only halophilic archaea the complete gene sequence of which has been published. No homologues of ostA and ttgR, however, were not detected in the genomes of either NRC-1 or any other archaea by FASTA and BLAST searches. Although the type strain of *Hbt. salinarum* used in this work is JCM 8978 (=NRC 34002), this strain is believed to be closely related to the strain NRC-1. Possibly, the mechanism of organic solvent tolerance of archaea may be different from those in bacteria.

The fact that strains OHF-1 and 2 are tolerant to two different extraordinary environmental factors, high salt concentrations and organic solvents, may be useful not only for industrial applications but for investigating the mechanism of how the halophilic archaea acquired the tolerance.

Acknowledgments

The authors thank Dr. Kamekura of the Noda Institute for Scientific Research for supplying some strains used in this study and for critical reading of the manuscript.

References